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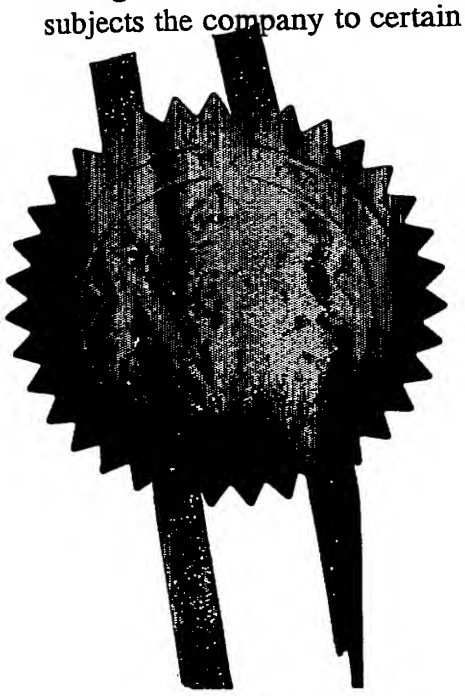

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Dated 7 September 2004

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1/77

020EP03 E966474-1 000866
P01/7700 0.00-0327900.7

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1.	Your reference GB Case	BT/3-22348/P1
2.	Patent application number *(The Patent Office will fill in this part)*	0327900.7 - 2 DEC 2003
3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	Ciba Specialty Chemicals Water Treatments Limited Cleckheaton Road Low Moor Bradford West Yorkshire BD12 0JZ
	Patent ADP number *(if you know it)*	7585391005.
	If the applicant is a corporate body, give the country/state of its incorporation	England
4.	Title of invention	PROCESS FOR PREPARING UNSATURATED AMIDES AND CARBOXYLIC ACIDS
5.	Name of your agent *(if you have one)*	
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Ciba Specialty Chemicals Water Treatments Limited Patents Department PO Box 38 Cleckheaton Road Low Moor Bradford West Yorkshire BD12 0JZ
	Patents ADP number *(if you know it)*	07585391002 ✓

		Country	Priority application number *(if you know it)*	Date of filing (day/month/year)
6.	If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number			

		Number of earlier application	Date of filing (day/month/year)
7.	If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application		

8.	Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *(see note (d))*	YES

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Continuation sheets of this form	-
Description	11 ✓
Claim(s)	2 ✓
Abstract	1 ✓
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Priority documents	-
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	-
Request for preliminary examination and search *(Patents Form 9/77)*	1
Request for substantive examination *(Patents Form 10/77)*	-
Any other documents *(please specify)*	-

11. I/We request the grant of a patent on the basis of this application

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Date 02 December 2003

12. Name and daytime telephone number of person to contact in the United Kingdom **Jane Spinks** **01274 417558**

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DUPLICATE

Process for Preparing Unsaturated Amides and Carboxylic Acids

The present invention relates to processes for preparing ethylenically unsaturated carboxylic acids from the corresponding nitriles or amides employing nitrilase or amidase respectively and for preparing ethylenically unsaturated amides from the corresponding nitriles employing nitrile hydratase. The invention also concerns new nitrile hydratase, amidase and nitrilase enzymes and microorganisms that can produce such enzymes.

The enzymic catalysis of chemical reactions is well-documented in the literature. It is well known to employ biocatalysts, such as microorganisms that contain enzymes, for conducting chemical reactions, or to use enzymes that are free of microorganisms. It is known that various ethylenically unsaturated monomers can be prepared by converting a substrate starting material into the desired monomer by use of a biocatalyst.

Nitrile hydratase enzymes are known to catalyse the hydration of nitriles to the corresponding amides. Typically nitrile hydratase enzymes can be synthesized by a variety of microorganisms, for instance microorganisms of the genus Bacillus, Bacteridium, Micrococcus, Brevibacterium, Corynebacterium, Pseudomonas, Acinetobacter, Xanthobacter, Streptomyces, Rhizobium, Klebsiella, Enterobacter, Erwinia, Aeromonas, Citrobacter, Achromobacter, Agrobacterium, Pseudonocardia, Rhodococcus and Comomonas.
It is known to produce acrylamide and ammonium acrylate from acrylonitrile on an industrial scale using as a catalyst nitrile hydratase and nitrilase respectively. When producing these products biologically it is desirable to employ an enzyme which is capable of producing aqueous solutions of acrylamide or ammonium acrylate in high concentration and yet is not poisoned by acrylonitrile and high concentrations of acrylamide or ammonium acrylate.

A review paper by Yamada and Kobayashi, Biosci. Biotech. Biochem 60: 1391-1400 (1996) charts the development of the biocatalysed process for the production of acrylamide monomer up to a concentration of 50%. This review describes the three generations of catalyst developed for the industrial production of acrylamide culminating with Rhodococcus rhodochrous J1, a bacterium that requires cobalt as part of the nitrile hydratase enzyme which catalyses the formation of acrylamide from acrylonitrile. The nitrile hydratase is synthesised in very high levels in the bacterium due to the presence of urea as an inducer in the culture medium.

A paper by Nawaz et al., Arch. Microbiol. 156:231-238 (1991), entitled 'Metabolism of acrylonitrile by Klebsiella pneumoniae' describes the isolation and growth of the bacterium K. pneumoniae and its subsequent rapid utilisation of acrylonitrile and formation of acrylamide which was then further hydrolysed to acrylic acid. The organism was isolated using an enrichment culture technique with acrylonitrile as the sole nitrogen source at pH 7.5.

Takashima et al., J Indust. Microbiol. Biotechnol. (1998), Nitrile hydratase from a thermophilic Bacillus smithii, describes the characteristics of a thermophilic bacterium which synthesises nitrile hydratase. The nitrile hydratase has high acrylonitrile converting activity and the highest activity was at pH 10.6 or above. This would suggest for optimum activity to be achieved for this enzyme, the reaction solution would have to be buffered at this high pH.

Ramakrishna and Desai Biotechnol. Lett. 15: (3) 267-270 (1993) describes the superiority of cobalt induced acrylonitrile hydratase of Arthrobacter sp. IPCB-3 for conversion of acrylonitrile to acrylamide compared with an iron containing nitrile hydratase in this organism. This bacterium requires cobalt, and urea as a co-factor and inducer respectively to give the highest nitrile hydratase activity. Although the cobalt containing nitrile hydratase of this organism appears to have good acrylonitrile tolerance, at acrylamide concentrations of greater than

25% the enzyme activity was greatly reduced.

Various strains of the Rhodococcus rhodochrous species have been found to very effectively produce nitrile hydratase enzyme. EP-0 307 926 describes the culturing of Rhodococcus rhodochrous, specifically strain J1 in a culture medium that contains cobalt ions. The nitrile hydratase can be used to hydrate nitriles into amides, and in particular the conversion of 3-cyanopyridine to nicotinamide. This organism is further described in EP-0362829, which describes a method for cultivating bacteria of the species Rhodococcus rhodochrous comprising at least one of urea and cobalt ion for preparing the cells of Rhodococcus rhodochrous having nitrile hydratase activity. Specifically described is Rhodococcus rhodochrous J1.

Rhodococcus rhodochrous J1, is used commercially to manufacture acrylamide monomer from acrylonitrile and this process has been described by Nagasawa and Yamada Pure Appl. Chem. 67: 1241-1256 (1995).

Leonova et al., Appl. Biochem. Biotechnol. 68: 231-241 (2000) entitled, "Nitrile Hydratase of Rhodococcus", describes the growth and synthesis of nitrile hydratase in Rhodococcus rhodochrous M8. The NH synthesis of this strain is induced by urea in the medium, which is also used as a nitrogen source for growth by this organism. Cobalt is also required for high nitrile hydratase activity. This literature paper mainly looks at induction and metabolic effects.

It is also known to produce ammonium acrylate directly from acrylonitrile by the action of a nitrilase enzyme. WO9721827 describes producing a concentrated solution of ammonium (meth) acrylate which is substantially free of (meth) acrylonitrile by the enzymic hydrolysis of (meth) acrylonitrile in the presence of water using a nitrilase enzyme which has a Km for (meth) acrylonitrile of below 500 micro moles and Ki for ammonium (meth) acrylate above 100,000 micro

moles. The enzyme can be obtained from a Rhodococcus rhodochrous microorganism.

Nagasawa et al., Appl. Microbiol. Biotechnol. 34:322-324 (1990) also describe the use of the nitrilase of Rhodococcus rhodochrous J1 for the synthesis of acrylic and methacrylic acid. They looked at the effects of temperature, acrylonitrile concentration and pH conditions on the reaction.

Each of the aforementioned references describe bacteria that produce nitrile hydratase or nitrilase enzymes. All of these disclosures require that the bacteria are grown at approximately neutral pH.

The genus Dietzia was first described by Rainey et al., Int. J. Syst. Bacteriol 45: 32-36 (1995). Dietzia maris became the type species for the genus. In 1999 a further species addition was made: Dietzia natronolimnaea, this species first being described by Grant et a., Extremophiles 2: 359-366 (1998) in the publication entitled 'Dietzia natronolimnaios sp. Nov., a new member of the genus Dietzia isolated from an East African soda lake'.

The Dietzia natronolimnaios strain isolated by these researchers 15LN1 (CBS 107.95) is an alkaliphile and as such grows at high pH (10) and in addition it grows in culture media containing high salt concentrations (40 g/l).

The Dietzia genus has been described for the catalysis of the synthesis of saturated compounds. For instance, WO-A-02/12530 describes a process for preparing 3-hydroxycarboxylic acid by the hydrolysis of 3-hydroxynitrile using Dietzia sp. ADL1 (ATCC PTA-1854).

A process for the preparation of glycine from glycinonitrile using microorgansims is described in WO01/048234. A number of microbial species are described in this patent including Dietzia maris.

Microorganisms which specifically produce acrylonitrilase or acrylonitrile hydratase enzymes or other analogous enzymes for converting unsaturated nitriles to the corresponding amides or carboxylic acids, are grown at about neutral pH, that is approximately pH 6 to 8. Consequently, it can be more difficult to maintain the sterility during the culturing of the bacterium as it is recognized that many, many microorganisms will grow at this pH. A particular problem that can occur therefore, is that the fermentation can become contaminated with other microorganisms. Such contamination not only impairs the production of the required enzyme, but may also result in undesirable by-products when used to convert the unsaturated nitrile to the desired product. Additionally it is most undesirable to have other microorganisms present as in order to ensure they are not harmful, that is not pathogenic, the unknown contaminants would have to be identified. Consequently, the fermentation would have to be abandoned which is both expensive and time-consuming.

It has already been described that urea is often added to the fermentation medium as an inducer of the nitrile hydratase of many organisms that are shown to produce acrylamide from acrylonitrile. Solutions of urea can be alkaline due to the presence of ammonium ion in the solution. And additionally if the urea is degraded at all during the fermentation this releases ammonium ion causing the pH of the medium to increase, unless buffering capacity in the form of buffer salts is added at high levels, or more likely the increasing pH effects are counteracted by the use of acid addition to the fermentation. This is therefore a further problem with fermenting the microorganism at neutral pH in that it is normally required to buffer the reaction mixture in order to counteract the effect of adding urea. Buffer solutions that are used may include phosphate salts, citric acid in combination with a basic salt such as phosphate, tris or any other buffer generally known to be applicable to use in fermentation systems to give rise to a neutral pH.

A further problem is that the known microorganisms tend not to be tolerant to high salt concentrations and this can result in cell leakage during growth and during use of the bacteria as a biocatalyst due to the differences in the osmotic pressure within the cell and in the fermentation or reaction medium. For instance if a microorganism is being used to prepare a carboxylic acid salt, this solution would have a higher ionic strength than water or buffer solution. It might be the case that dependent upon the microorganism used, the difference in the osmotic pressure in the cell and the reaction solution would cause the cell to rupture thus reducing the capability of the organism to act as an effective biocatalyst and also by virtue of the intracellular material now being present as a contaminant of the reaction mixture, which may be wholly undesirable.

It would therefore be desirable to provide a process and a biocatalyst which overcomes all of these problems. In particular it will be desirable to provide a process in which ethylenically unsaturated carboxylic acids and their ammonium salts can be prepared from the corresponding nitrile or amide and in which ethylenically unsaturated amides are prepared from the corresponding nitrile in high yield and without the risk of unwanted byproducts.

In accordance with the first aspect of the present invention we provide a process of producing an ethylenically unsaturated amide, wherein a nitrile is treated with an enzyme which is a nitrile hydratase in an aqueous medium, characterised in that
the nitrile hydratase is obtainable from a microorganism of the Dietzia genus.

According to the second aspect of the present invention we provide a process of producing an ammonium salt of an ethylenically unsaturated carboxylic acid, wherein a nitrile is treated with an enzyme which is a nitrilase in an aqueous medium, characterised in that
the nitrilase is obtainable from a microorganism of the Dietzia genus.

According to the third aspect of the present invention we provide a process of producing an ammonium salt of an ethylenically unsaturated carboxylic acid, wherein an amide is treated with an enzyme which is an amidase in an aqueous medium, characterised in that
the amidase is obtainable from a microorganism of the Dietzia genus.

Unexpectedly we have found that microorganisms of the Dietzia genus are capable of producing specific nitrile hydratase, amidase and nitrilase enzymes suitable for converting ethylenically unsaturated nitriles to the corresponding amides and carboxylic acids on an industrial scale.
The Dietzia microorganisms can be cultured at alkaline pHs, for instance pH 9 to 10 which facilitates improved sterility of the fermentation. Advantageously we now find that the fermentation and bio-process can be integrated and thus carried out in a single step.

Furthermore, the Dietzia microorganisms have unexpectedly been found to exhibit high tolerance to high concentrations of unsaturated carboxylic acids and therefore enable ethylenically unsaturated nitriles, such as acrylonitrile, to be converted into the carboxylic acid, such as acrylic acid (as the ammonium salt) at high concentrations. Typically such high concentrations of acids could be expected to bring about cell leakage and render them unsuitable for re-use in a bioconversion process. It is also possible to use the Dietzia microorganisms and nitrile hydratase produced therefrom to produce acrylamide in high concentrations. Furthermore it is also possible to synthesise ethylenically unsaturated carboxylic acid in high concentrations from the corresponding amide using Dietzia and amidase enzyme produced by the Dietzia microorganisms. In a further development of the process ethylenically unsaturated carboxylic acids can be prepared from the corresponding nitrile in a two-step process involving a first stage hydration to the corresponding amide using the nitrile hydratase of Dietzia and a second stage conversion of the amide into the carboxylic acid using the amidase produced by Dietzia.

Preferably the ethylenically unsaturated nitrile is (meth) acrylonitrile, the ethylenically unsaturated amide is (meth) acrylamide and the ethylenically unsaturated carboxylic acid is (meth) acrylic acid.

In each case the enzymes may be extracted from the microorganism and used directly in the reaction. Preferably though the enzymes are comprised within whole cells of the microorganism.

The microorganism may be any species of the Dietzia genus but is preferably a species of Dietzia selected from the group consisting of Dietzia natronolimnaios, Dietzia maris and Dietzia psychralcaliphila.

Most preferably the microorganism used to provide the nitrile hydratase or,amidase or nitrilase enzymes is a new microorganism Dietzia natronolimnaios strain 2347 (NCIMB 41165). In a further aspect of the present invention we claim the new microorganism Dietzia natronolimnaios strain 2347.

The nitrile hydratase enzyme, the amidase enzyme and the nitrilase enzyme each obtainable by culturing Dietzia natronolimnaios strain 2347 are also new. The details of strain 2347 are given below:

1. Origin and Deposition

The strain 2347 was isolated by us from soil in Bradford, England and deposited on 5th March 2003 at the National Collection of Industrial and Marine Bacteria (NCIMB), where it was assigned the accession number NCIMB 41165 under the Budapest Treaty.

2. Morphological and cultural characteristics

(1) Polymorphic growth

(2) Motility: immotile

(3) Non-spore former

(4) Gram positive

(5) Aerobic

(6) Growth on nutrient agar gives pink round shiny colonies within 48 hours at 30°C

(7) Growth on Alkaline Medium yields bright red shiny colonies with mucous texture.

3. Cultivation and Nitrile Hydratase Synthesis

The Dietzia bacteria, for instance Dietzia natronolimnaios, of the present invention can be cultivated under any conditions suitable for the purpose, but it is most preferable to grow in a medium that is alkaline and which may also contain salt at high levels. Examples of suitable culture media are shown in the patent examples.

In addition a suitable inducer for the nitrile hydratase, amidase or nitrilase should be included in the growth medium. These could be a nitrile such as acetonitrile, propionitrile, isobutyronitrile or acrylonitrile or an amide such as acetamide, propionamide, isobutyramide or acrylamide. Specifically for nitrile hydratase activity for amide formation, urea could be used as enzyme inducer.

The following examples illustrate the invention.

Example 1

A) Dietzia natronolimnaios NCIMB 41165 was isolated from soil and it was grown in a 2L baffled Erlenmeyer flask containing 400 mL culture medium containing the following constituents (g/L): diPotassium hydrogen phosphate 0.7; Potassium hydrogen phosphate 0.3; glucose 10.0; yeast extract 3.0; peptone 5.0; magnesium sulphate heptahydrate 0.5; Urea 5.0; cobalt chloride hexahydrate 0.01; tap water to 1L. The pH of the medium was adjusted to pH 7.2. The culture was grown at 28°C for 5 days. Biomass was harvested by centrifugation and stored at –20°C.

B) Dietzia natronolimnaios NCIMB 41165 was grown as described in (A). However the urea was removed from the medium and acetonitrile was added at 5 g/L.

C) Dietzia natronolimnaios NCIMB 41165 was grown as described in (A). However the urea was removed from the medium and isobutyronitrile was added at 5 g/L

D) A portion of the biomass from (A) was defrosted and suspended in 50mM pH 7 sodium phosphate buffer (20 mL). The suspension was incubated at 15°C for 10 minutes. Acrylonitrile (0.247 mL) was added to the cell suspension and the mixture was shaken. A sample (0.3 mL) was removed immediately and it was added to a solution of 8.8% o-phosphoric acid (0.3 mL). The cells were removed by centrifugation. The supernatant was analysed by HPLC for the presence of acrylonitrile, acrylamide and ammonium acrylate. The reaction was carried out for 10 minutes. The specific nitrile hydratase activity of the cells was 44,170 µmoles/minute/g dry weight of cells.

A portion of the biomass from (B) was treated as described in (D). The specific nitrile hydratase activity of the cells was 11,060 µmoles/minute/g dry weight of cells.

E) A portion of the biomass from (C) was treated as described in (D). The specific nitrile hydratase activity of the cells was 1150 µmoles/minute/g dry weight of cells.

Example 2

A) Dietzia natronolimnaios NCIMB 41165 was grown for 5 days at 30°C in the following culture medium in g/L: peptone 5.0; yeast extract 5.0; glucose 10; potassium dihydrogen phosphate 1.0; magnesium sulphate heptahydrate 0.2; sodium chloride 40.0; disodium carbonate 10.0. pH 10.0

B) Dietzia psychralcaliphilia NCIMB 13777 was grown in the medium described in (A)

Claims

1. A process of producing an ethylenically unsaturated amide, wherein a nitrile is treated with an enzyme which is a nitrile hydratase in an aqueous medium, characterised in that
the nitrile hydratase is obtainable from a microorganism of the Dietzia genus.

2. A process of producing an ammonium salt of an ethylenically unsaturated carboxylic acid, wherein a nitrile is treated with an enzyme which is a nitrilase in an aqueous medium, characterised in that
the nitrilase is obtainable from a microorganism of the Dietzia genus.

3. A process of producing an ammonium salt of an ethylenically unsaturated carboxylic acid, wherein an amide is treated with an enzyme which is an amidase in an aqueous medium, characterised in that
the amidase is obtainable from a microorganism of the Dietzia genus.

4. A process according to claim 1 or claim 2 in which the ethylenically unsaturated nitrile is (meth) acrylonitrile.

5. A process according to any of claims 1 to 4 in which the ethylenically unsaturated amide is (meth) acrylamide.

6. A process according to any of claims 2 to 5 in which the ethylenically unsaturated carboxylic acid is (meth) acrylic acid.

7. A process according to any of claims 1 to 6 in which the enzyme is comprised within whole cells of the microorganism.

8. A process according to any of claims 1 to 7 in which the microorganism is a species of Dietzia selected from the group consisting of Dietzia spp., Dietzia natronolimnaios, Dietzia maris and Dietzia psychralcaliphila.

9. A process according to any of claims 1 to 8 in which the microorganism is Dietzia natronolimnaios strain NCIMB 41165.

10. Dietzia natronolimnaios strain NCIMB 41165.

11. Nitrile hydratase enzyme obtainable by culturing Dietzia natronolimnaios strain NCIMB 41165.

12. Nitrilase enzyme obtainable by culturing Dietzia natronolimnaios strain NCIMB 41165.

13. Amidase enzyme obtainable by culturing Dietzia natronolimnaios strain NCIMB 41165.

5

Abstract

Process for Preparing Unsaturated Amides and Carboxylic Acids

A process of producing an amide from a nitrile by the action of a nitrile hydratase enzyme or ammonium salt of an ethylenically unsaturated carboxylic acid from a nitrile or an amide by the action of a nitrilase or amidase respectively in which enzymes are obtainable from a microorganism of the Dietzia genus.